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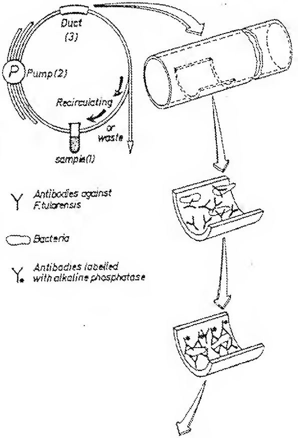
(54) Title: A METHOD OF CONCENTRATING AND DETECTING BIOMOLECULES AND -CELLS AND A MEANS THEREFORE

(57) Abstract

A method of detecting biological substances having affinity properties by passing the substance to be detected in a flow over a solid surface to which surface a second substance, to which the substance to be detected shows affinity, is attached, so that the two substances form a complex and give enrichment of the substance to be detected. The enrichment is obtained when the fluid volume passing the active surface is many times larger than the volume in contact with the active surface. A means for detecting biological substances consisting of a flow of a fluid sample of the substance to be detected generated via a pump over a solid surface to which another substance, to which the first substance to be detected shows affinity, is attached, so that a complex is formed of the two substances and gives enrichment of the substance to be detected.



A method of concentrating and detecting biomolecules and -cells and a means therefore.

This invention relates to a method and a means for concentrating and detecting biological material.

Previously known methods for assaying biological and biochemical materials have been unsatisfactory as it has often been difficult to concentrate said material for assay. Different techniques, such as spin-drying and ultrafiltration, to obtain an increased concentration of the biological and biochemical material to be analysed have been used. However, it appeared that several molecules could not be concentrated in such a way and examples thereof are antigens, nucleic acids, hormones, enzymes, ligands, etc. A serious drawback with said techniques is that also contaminating molecules are concentrated and interfere with the detection of the desired material.

The present invention relates to a method for separately concentrating biological and biochemical materials having affinity properties. This is achieved by letting, in a flow, a fluid sample of the material to be detected and which material has affinity to another material, such as antigen-antibody, DNA-DNA, DNA-RNA, RNA-RNA, lectin-receptor, ligand-interactions as well as other receptors, e.g. phages and viruses, pass a solid surface, at which surface the other material to which the material to be detected has affinity is attached, so that in relation to the volume of the fluid a many times larger volume passes the surface. During the passage of the flow, the material to be detected adheres to the other material to which it has affinity and forms a

complex, which can be read off by different markers such as enzymes, radio activity, laser, etc.

The detection process according to this invention can be automatized and the sensitivity increased at least ten times as compared to previously known methods such as "enzyme-linked immunosorbent assay" (ELISA) with microplates or by radioimmunoanalysis (RIA) techniques, immunofluorescence, etc. (Voller, A., Bartlett, A., and Bidwell, D.E., "Enzyme immunoassays with special reference to ELISA techniques", J. Clin. Pathol. 31, 507-520 (1978); Overby, L.R., and Mushahwar, J.K., "Radioimmune assays", p. 39-70, in M.W. Rytel (Ed.), Rapid diagnosis in infectious disease, CRC Press, Boca, Fla. (1979); Dahle, A.B., and Laake, M., "Diversity dynamics of marine bacteria studies by immunofluorescent staining on membrane filters", Appl. Environ. Microbiol. 43, 169-176 (1982).

Depending on the flow ratio and the flow time, a considerable lower limit for the detectable level is obtained. The concentration of bacteria and the amounts of the bound substances are other variables. The extent of bound substance is increased with increased specificity.

The amount of flow must be tested each time and depends *inter alia* on the size of the molecules to be detected.

The solid surface can be any solid surface which is immobilized, but is preferably hydrophobic and consists of polymeric substances such as glass, plastic material, metal, silicon, etc. The bindning to said surface is obtained

1) by passive adsorption to the hydrophobic surface, or

2) by immobilizing the specific molecule, e.g. antibody, enzyme, antigen, to a solid phase by adsorption or covalent binding.

The fluid sample can consist of water, water-based systems, e.g. buffer, body fluids, in cyclon media impacted air samples, in suitable buffer systems re-suspended solid samples containing the substance to be detected.

In bacteria assay it has been shown to be advantageous if the sample contains at least 10^3 bacteria per milliliter suspended therein, but also as small amounts as 10^2 bacteria per milliliter could be detected.

The concentration of the desired substance can be decreased if the volume of the sample is increased and yet give detectable results.

A non-limiting example of a means used according to the present invention is shown in the enclosed schematical view of the concentration process by means of immunological technique (Figure 1).

A sample (1) containing for example antigen is pumped by a pump (2) into a duct (3) to the inner surfaces of which antibodies are bound. By the flow through the duct antigens are deposited on the antibodies and form a complex which is read off by different markers, e.g. a colour reaction with alkaline phosphatases.

Hitherto used assay methods such as the above mentioned ELISA-methods have restrictions as regards the lowest amounts of detectable antigen (sensitivity). Different direct concentration increasing measures such as centrifugation and ultrafiltration have, as stated above, been used to increase the concentration and sensitivity without giving satisfactory results.

Antibodies obtained from rabbits which had obtained antigens from Francisella tularensis (the causative agent of tularemia) were tested regarding their ability to recognize whole

bacteria of *F. tularensis*. It was then found that the antibodies were bound to the surfaces of the bacteria and could be used as a diagnostic means. An assay system was developed based on said antibodies in which the marker antibodies were affinity purified against the antigens and labelled with alkaline phosphatase.

When said diagnostic system was tested with whole bacteria of *F. tularensis* by so-called microplate-ELISA, it was found that the lowest amount of bacteria that significantly could be detected by said method was at a concentration of 10^5 bacteria per milliliter (Figure 2).

Concentration by centrifugation or ultrafiltration gave the same result on analysis (i.e. no increase was obtained, not shown).

Investigations of epidemics of tularemia have shown that *F. tularensis* can be present in and spread by water and air. A demand for assay of water and air containing minor amounts of bacteria has thus arisen. Another existing demand is also to assay very small samples, such as body fluids.

As mentioned above, the ELISA-methods as used proved to be unsatisfactory, as the lowest amounts of bacteria that can be detected by said methods are within a concentration of 10^5 bacteria per milliliter.

Therefore, tests were made using the affinity of *F. tularensis* to specified antibodies to increase the sensitivity of the test.

A surface antigen was prepared as described by Sandström, G., Tärnvik, A., Wolf-Watz, H., and Löfgren, S., "Preparation of antigen from *Francisella tularensis* for demonstration of antibodies by the ELISA", FOA Report C 40179-B 3, June 1983, Umeå, Sweden.

A surface antigen (Sandström et al., Inf. Imm. 45, 101-106 (1984)) was isolated which immunized on rabbit gave specific antibodies against *F. tularensis* (Sandström, G., and Wolf-Watz, H., "Rapid identification of *Francisella tularensis* in water", FOA Report C 40188-B 3, November 1983, Umeå, Sweden.

Rabbit sera

Rabbits were immunized by *F. tularensis* antigen as described by Sandström, G., and Wolf-Watz, H., "Rapid identification of *Francisella tularensis* in water", FOA Report C 40188-B 3, November 1983, Umeå, Sweden.

Affinity purification and alkaline phosphatase-coupling to rabbit antibodies

A live vaccine strain of *F. tularensis* (*F. tularensis* LVS) was provided by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md., USA. A wild strain of *F. tularensis var. palaerctica* (strain SBL R45) was provided by R. Möllby, The National Swedish Bacteriological Laboratory, Stockholm, Sweden.

Bacteria of two strains of *F. tularensis* were grown on modified Thayer-Martin agar containing Gc-medium base (36 grams/liter; Difco Laboratories, Detroit, Mich., USA), hemoglobin (10 grams/liter; Difco) and IsoVitalex (10 milli-grams/liter; BBL Microbiology Systems, Cockeysville, Md., USA) at 37°C in 5% CO_2 in air. The bacteria population was determined by counting live bacteria.

Fluid test:

Tap water was adjusted to pH 5.0 using 0.01M hydrochloric acid. Tween® 20 was added to each sample at a concentation of 0.05% (v/v). *F. tularensis* bacteria were suspended in amounts

of 0, 10^1, 10^2, 10^3, 10^4 and 10^5/ml, respectively, or alternatively, cyclon medium was supplemented with 0.05% (v/v) Tween® 20 (Olsson, T., Stymne, S., and Thore, A., "Detektion av bakterieaerosoler med luminescensanalys 1. Luminescens-analys av luftprover", FOA Report C 40061-B 2 (1977), Ursvik, Sweden.

Reference Example

Antigen - antibody

Microplate ELISA was performed substantially according to Voller, et al., "Enzyme immuno assays with special reference to ELISA techniques", J. Clin. Pathol., 31, 507-520 (1978). Rabbit antiserum against F. tularensis-antigen diluted 100:1 (ELISA-titer 1:5000) in 0.05M sodium bicarbonate buffer pH 9.6 was coated on microplates (Flow Laboratories Svenska AB, Stockholm, Sweden). Bacterial suspensions were added to the microplates which were incubated for one hour at 37°C. In the next step, a solution of affinity-purified, alkaline phosphatase labelled rabbit antibodies against F. tularensis antigen was applied for one hour at 37°C. The reaction developed by the addition of the substrate to the enzyme and the absorbance at 405 nm was measured. The results thus obtained are shown in Figure 2 and Table 2.

Example

Antigen - antibody

The process was performed in plastic tubes (Tygon®; Noax AB, Stockholm, Sweden) connected to a peristaltic pump system (Technicon Corp., Ardesly, USA). The volume of the tube was 40 µl/cm tube. For each assay 10 cm of tube was connected. Antiserum in a stationary condition was coated over night at room temperature. Test samples were passed through the tube at a flow rate of 1.9 ml/min. (figure 3).

In some experiments test samples were stationary in the tube while during other tests a flow was generated through the tube.

After washing, stationary exposure to alkaline phosphatase-labelled antibodies was performed at 37°C for one hour. The tubes were disconnected from the pump system and cut from each side to a length of 5 cm, and substrate to alkaline phosphatase was added. After incubation at 37°C for 30 minutes the content of each tube (200 µl) was transferred to the well of a microplate and the absorbance at 405 nm was measured. The samples were tested in triplicate and the mean values were calculated.

When the test samples were stationary in the duct, lower ELISA values were obtained in relation to the values obtained when the samples were flown through the duct (Figure 3).

By using the process according to the present invention, an increased sensitivity was obtained. With an unlimited test sample it is possible to detect as few as 10^2 F. tularensis per milliliter (Figure 4). Viable counts of F. tularensis before and after the passage through the duct did not show any measurable decrease in bacteria.

The possibilities of recirculating different volumes of test samples containing F. tularensis were tested. The smallest volume that for practical reasons could be recirculated was a 5 ml test sample. The sensitivity was increased at least 10 times (Figure 5), generally between 10-100 times, in relation to the time during which the test sample was recirculated (Figure 4). By circulating 10^4 F. tularensis bacteria suspended in 5, 15, and 50 ml of fluid test samples for 18 hours increased values were obtained depending on the amounts of fluid sample as tested (Table 1).

When different volumes of the sample containing F. tularensis were flown through the duct coated with antibodies specific against F. tularensis, an enrichment in bacteria in the duct was obtained. When larger volumes were passed through the duct, a plateau level was obtained (Figure 3), probably due to the fact that all antigen deposition sites were already occupied and thus no further bacteria could be trapped.

Similar results were obtained when F. tularensis were impacted in cyclon media supplemented with 0.05% (v/v) Tween® 20 (Olsson, T., Stymne, S., and Thore, A., "Detektion av bakterieaerosoler med luminescensanalys 1. Luminescensanalys av luftprover", FOA Report C 40061-B 2 (1977), Ursvik, Sweden.

Ligand-ligand-interaction

E. coli (HB101/pRHU845) agglutinates A blood via mannose-resistant hemagglutination. Interaction occurs between a special protein structure of the bacteria to a Gal-NAc ß (1 → 3) Gal 2 (1 → 4) Gal ß (1 → 4) GLC-ceramide receptor on red blood corpuscles.

A plastic tube (Tygon®) was coated with whole blood (A-blood). E. coli bacteria in concentrations of from 0 to 5.10^6 bacteria per milliliter were flooded through the tube system. Thereafter rabbit antibodies against E. coli were added. Then anti-rabbit IgG labelled with alkaline phosphatase were added for one hour at 37°C and finally the absorbance at 405 nm was monitored by the addition of substrate to alkaline phosphatase. Using this method it was possible to detect E. coli at different densities.

Table 1

Results obtained when 10^4 *F. tularensis* were circulated in varying amounts of fluid test sample for 18 hours.

Volume[a] (ml)	A 405/100 minutes: 0 F.tul/ml	A 405/100 minutes: 10^4 F.tul/ml
5	0.57 ± 0.06	0.87 ± 0.13
15	0.60 ± 0.07	1.13 ± 0.20
50	0.68 ± 0.05	1.27 ± 0.23

[a] Recirculated for 18 hours
A405/100 min. = absorbance at 405 nm/100 min.
The results are mean values of 3 measurements.

Table 2

Results obtained for tests using microplate ELISA, assay with the fluid stationary in the duct and assay with a flow

Amount of bacteria per ml	Microplate ELISA	Fluid stationary in the duct (Tygon® plastic tube)	Fluid in flow (Tygon® plastic tube)
10^5	0.125[a]	0.335	0.504
10^4	0.025	0.086	0.162
10^3	0.000	0.027	0.064

[a] Absorbance at 405 nm/100 min. The values are decreased by the values obtained for tap water without *F. tularensis.*

The level of detectable F. tularensis LVS was increased by using the fluid in flow. Suspensions containing 10^3, 10^4 and 10^5 F. tularensis cells per milliliter were passed through a Tygon® plastic tube coated with antibodies against F. tularensis. The flow rate of the test suspension was about 100 ml per 3 hours. According to this process the amount of F. tularensis LVS was enriched and the detectable level became lower than the detectable level obtained with microplate ELISA.

The influence of Tygon® plastic tube was examined. The ELISA assay was performed without any flow in the Tygon® plastic tube. The advantages of the fluid in flow where shown also in this test. A lower detectable level was obtained as compared to microplate ELISA.

LEGENDS TO FIGURES

Fig. 1: Enrichment of the duct walls using a flow system. The flow rate was 1.9 ml/min. and the volume 40 µl/cm in the duct. The test sample was either flown straight through the duct or recirculated. The assay was performed in the same way as the microplate ELISA assay with respect to antigen and antibody. The length of the duct was 10 cm during the process, except for the last step when enzyme substrate was added. Before said addition the duct was cut from both ends to a length of 5 cm.

Fig. 2: ELISA was performed in microplates. The microplates were pre-coated with immune serum against *F. tularensis* antigen at room temperature over night. The test samples were added after washing and allowed to react at 37°C for one hour. Affinity purified anti-*F. tularensis* antibodies labelled with alkaline phosphatase were subsequently added and the microplates were incubated for one hour at 37°C. Enzyme substrate was finally added and the reaction monitored after 30 minutes, using an automatic spectrophotometer. Stated values are mean values ± standard deviation of 16 samples.

Fig. 3: Enrichment of *F. tularensis* at the duct wall using different volumes of water containing 10^5 bacteria per milliliter. The test time was 3 hours. Stated values are mean values ± standard deviation of 3 samples.

Fig. 4: The detection level for different concentrations of *F. tularensis* depended on the volume of the test sample which passed through the pre-coated Tygon(R) tube. After 3 hours, 10^4 *F. tularensis* could be detected.

To obtain a significant detection level for as few as 10^2 bacteria per milliliter, about 4.5 l sample (a total of 4.5 x 10^5 bacteria) was allowed to pass the Tygon® tube.

Said test shows that antigen can be enriched at the inner wall of the Tygon® tube if said tube is coated with specific antibodies. It is also evident that the test time, i.e. the enrichment time, depends on the amount of bacteria in the sample to be tested.

Bacteria/ml	Graph plotting
10^2	○ - ○
10^3	▲ - ▲
10^4	■ - ■

Mean values ± standard deviation of 9-12 samples.

The background values (tap water without *F. tularensis*) are subtracted from the values as stated.

Fig. 5: Detection levels of *F. tularensis* when test samples are recirculated.

Bacteria/ml	Graph plotting
10^2	○ - ○
10^3	+ - +
10^4	■ - ■
10^5	▲ - ▲

The measurements were performed after 3, 6, 18 and 24 hours. Stated values are mean values ± standard deviation of 10-15 samples.

Fig. 6: Absorbance at 405 nm/100 min. at test with <u>E. coli</u> in concentrations between 0 and 5.10^6 per milliliter. The sample was recirculated for 3 hours. Volume = 5 milliliter. Stated values are mean values of 3 samples.

CLAIMS:

1. A method of detecting biological substances having affinity properties, characterized in that a fluid test sample containing the substance to be detected, which substance shows affinity to another substance, in a flow passes a solid surface to which surface the other substance, to which the first substance to be detected shows affinity, is attached, so that an enrichment of the substance to be detected is obtained and in relation to the fluid volume in contact with the active surface a many times larger fluid volume passes the surface.

2. A method according to claim 1, characterized in that the substance to be detected and the substance to which said first substance shows affinity consist of antigen-antibody, DNA-DNA, DNA-RNA, RNA-lectin receptor, ligand-interactions and other receptors such as phages and viruses.

3. A method according to claim 1, characterized in that the flow is generated by a pump.

4. A method according to claim 1, characterized in that the flow without being recirculated passes the solid active surface.

5. A method according to claim 1, characterized in that the flow is recirculated.

6. A method according to any of the preceding claims, characterized in that the flow rate is determined with respect to the size of the molecule to be detected.

7. A means for detecting biological substances having affinity properties, characterized by a flow over a solid surface of a fluid sample containing the substance to be detected which shows affinity to another substance, which latter substance is attached to the solid surface, whereby the

substance to be detected is enriched.

8. A means according to claim 7, characterized in that the solid surface is hydrophobic.

9. A means according to claim 7, characterized in that the flow is generated by a pump.

10. A means according to claims 7 and 8, characterized in that the solid surface consists of a polymeric material, e.g., glass, plastic material, metal or silicon.

11. A means according to any of claims 7, 8 and 10, characterized in that the solid surface consists of a flexible plastic tube.

Fig. 1

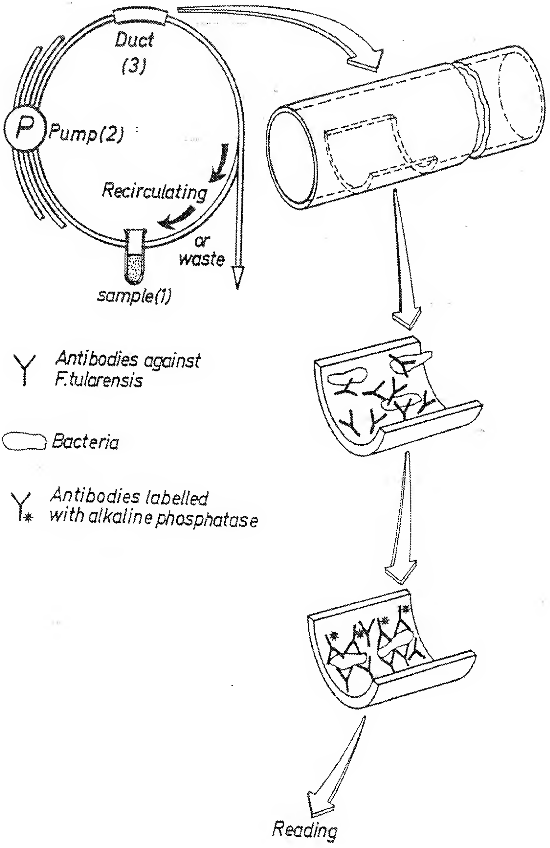

Duct
(3)
P
Pump(2)
Recirculating
or
waste
sample(1)
Antibodies against
F.tularensis
Bacteria
Antibodies labelled
with alkaline phosphatase
Reading

Fig. 2

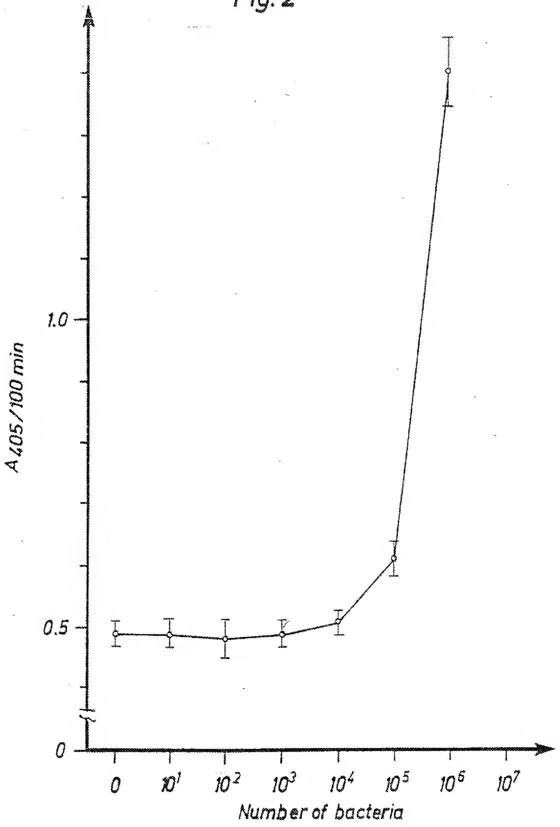

1.0
0.5
0
A 405/100 min
0
10¹
10²
10³
10⁴
10⁵
10⁶
10⁷
Number of bacteria

Fig. 3

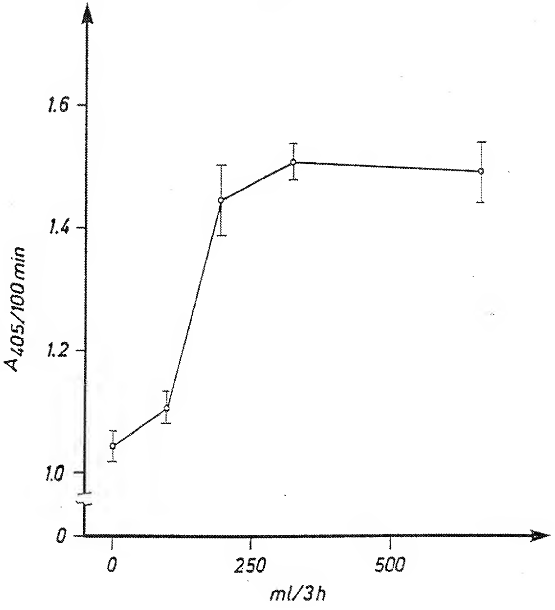


Fig.4

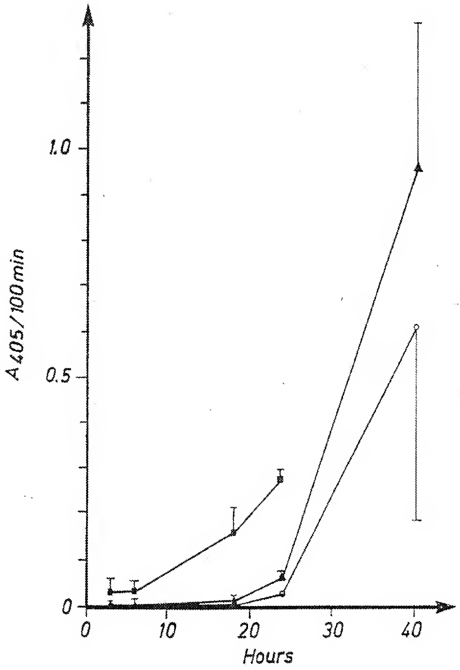


Fig.5

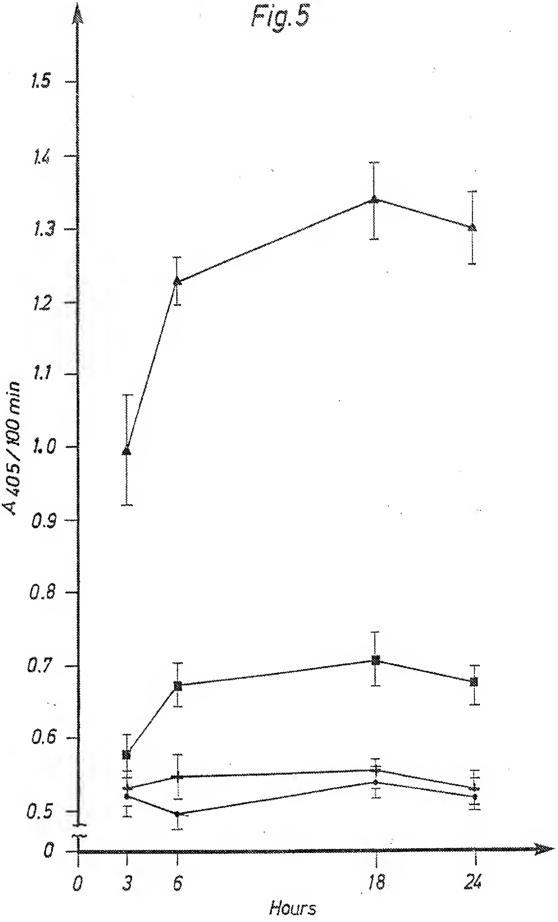
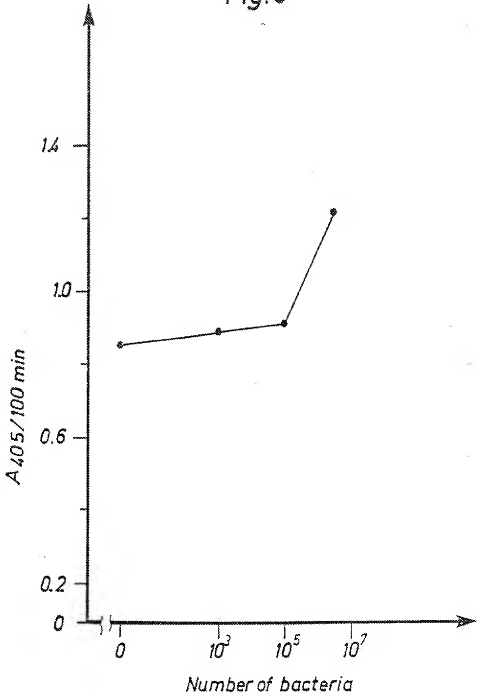


Fig. 6



INTERNATIONAL SEARCH REPORT

International Application No PCT/SE85/00026

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 4

G 01 N 33/53, C 12 Q 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC	G 01 N 33/54,56,53,537,538
US Cl	23:230B, 230.3, 253; 422:69, 70, 71; 424:1, 1.5; 435:4, 7; 436:518, 528; 531:35

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	Analytical Chemistry vol. 49 p. 1183 A-1190 A, published 1977 (Gochman N., Bowie L J) "Automated Systems for Radioimmunoassay" see in particular p. 1186 A - 1187 A	7-10
Y	DE, A, 3 145 007 (MAX-PLANCK GESELLSCHAFT) 19 May 1983 & EP, 0079466; JP, 58090518; AU, 90193/82	7-11
X	GB, A, 1 460 631 (SUMMA CORP) 6 January 1977 & NL, 7403215; FR, 2222654; DE, 2412833; US, 3896217; US, 4009005; NL, 7603439; FR, 2307268; DE, 2612948; US, 4059685; CA, 1036935	7-10

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* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
1985-03-29	1985-04-15
International Searching Authority	**Signature of Authorized Officer** [20]
Swedish Patent Office	Carl-Olof Gustafsson

VH

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	JP, 49125517 GB, 1527264 US, 4166102 JP, 51123819 US, 4180383 CA, 1077392 CA, 1083934 DE, 2660445 SE, 7604097 SE, 8000662 SE, 437193	
A	WO, A1, 83/01119 (COMMONWEALTH SERUM LAB. COMMISSION) 31 March 1983	1-11
A	FI, B, 60 617 (KOMMANDIITTIYHTIÖ FINNPIPETTE) 30 October 1981	1-11
A	FR, A, 2 498 331 (KADOUCHE J) 23 July 1982	1-11
A	FR, A, 2 389 134 (SEROA) 24 November 1978	1-11